



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/62, C07K 19/00	A1	(11) International Publication Number: WO 98/18943 (43) International Publication Date: 7 May 1998 (07.05.98)
(21) International Application Number: PCT/EP97/05897 (22) International Filing Date: 24 October 1997 (24.10.97) (30) Priority Data: 96810719.3 28 October 1996 (28.10.96) EP (34) Countries for which the regional or international application was filed: DE et al. (71) Applicants (for all designated States except US): NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH). UNIVERSITY OF LAUSANNE [CH/CH]; Institute of Biochemistry, Chemin des Boveresses 155, CH-1066 Epalinges (CH). SWISS INSTITUTE FOR ALLERGY AND ASTHMA RESEARCH [CH/CH]; Obere Strasse 22, CH-7270 Davos (CH). ISREC (INSTITUT SUISSE DE RECHERCHES EXPERIMENTALES SUR LE CANCER) [CH/CH]; Chemin des Boveresses 155, CH-1066 Epalinges (CH). (72) Inventors; and (75) Inventors/Applicants (for US only): TERSKIKH, Alexey [RU/CH]; Chemin des Bégonias, CH-1018 Lausanne (CH). CRAMERI, Reto [CH/CH]; Edenstrasse 13, CH-7270 Davos Platz (CH). MACH, Jean-Pierre [CH/CH]; Avenue de Valmont 20, CH-1010 Lausanne (CH). LE DOUSSAL,		Jean-Marc [FR/FR]; 20, rue Constantinople, F-75008 Paris Cedex (FR). KAJAVA, Andrey [RU/CH]; Chemin du Mont Tendre 20, CH-1007 Lausanne (CH). (74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <i>With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.</i> <i>Date of receipt by the International Bureau:</i> <i>15 January 1998 (15.01.1998)</i>
(54) Title: METHOD FOR THE OLIGOMERISATION OF PEPTIDES (57) Abstract <p>The present invention concerns an oligomer comprising more than 2 units, wherein each unit comprises a peptidic domain capable of oligomerizing and a domain capable of binding to an acceptor (ligand), wherein the oligomerizing domain is not an antibody or a functional antibody fragment from the constant region. Also described is the use and synthesis of this oligomer.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHOD FOR THE OLIGOMERISATION OF PEPTIDES

Great strides made over the past years in understanding of molecular interactions in the realm of biomolecules such as proteins and nucleic acids benefited from the isolation of artificial polypeptide "ligands" with *de novo* binding activities to "receptors". A powerful means of evolving artificial ligands is offered by the use of large polypeptide libraries, displayed on the surface of filamentous bacteriophage as a fusion with the phage coat proteins. In particular, isolation of new peptide ligands allowed, for example, to map antibodies binding sites, to find an important residues in HLA-DR molecules, to identify proteases substrates and inhibitors. Obviously, isolation of new peptide ligands with high affinity towards their target "receptors" can help to answer important biological questions and is of great interest for pharmaceutical development. However, apart from some exceptions, only low affinity (micromolar range) ligands were isolated from peptide libraries. This can be readily explained by a high degree of conformational freedom and a few number of contact residues within a short peptide molecule.

In order to improve this situation, a new type of high avidity binding molecule is constructed by harnessing the effect of multivalent interaction. In the current examples a short peptide ligand is expressed *via* a semi-rigid hinge region as a fusion with a coiled coil domain from Cartilage Oligomeric Matrix Protein (COMP), resulting in a pentameric multivalent binding molecule. In the case of a Pentabody (Pab-S) as described here, a peptide ligand (S) specific for the mouse B-cell lymphoma (BCL₁) surface idiotype, is selected from a phage displayed library. Pab-S molecule specifically bind the BCL₁ cells surface idiotype with an apparent avidity of about 1nM, which corresponds to a 2×10^5 fold increase, compared to the affinity of the synthetic peptide S itself. Equilibrium binding studies strongly suggest that high avidity of Pab-S is a result of multivalent interaction of its "peptide heads" and surface BCL₁ immunoglobulin receptor. As demonstrated by gel filtration chromatography, SDS-PAGE analysis and circular dichroism, Pab-S is a stable homopentamer of about 85kDa, with the five chains crosslinked by disulfide bridges. Pab-S can be reversibly denatured, reconstituting upon renaturation its pentameric structure and full binding activity, providing an easy way to bring different peptide specificity within the same heteropentamer, thus bispecific or multispecific pentabodies.

The inventive oligomers have various unique features.

- Target molecule specificity of inventive oligomer can be provided by a short peptide ligand, representing a "minimal" binding domain, where the primary structural information (i.e. the amino acid sequence) is sufficient for recognition. This demonstrates for the first time, that a low affinity peptide ligand (though specific) can be used to create a high avidity binding molecule.
- A hinge region which dictates the geometry and the dynamic features of multivalent interaction can be easily introduced.
- The inventive oligomers can be produced without extensive post translational modifications and, hence, can be produced easily in microorganisms like *E. coli*.
- The peptide ligands can be attached to the C- or N-terminus of the oligomerizing domain, thus providing various binding geometry, with maximal distance between two peptide ligands (for example some attached to the C-terminus and some to the N-terminus in one oligomer).
- If coiled coil α -helical domains are used, the resulting oligomers are in most cases well soluble due to the intrinsic structure.
- The COMP domain can be used generally for oligomerization of compounds bound thereto.
- It is a general method for the oligomerization of peptides.

Detailed description of the invention

The present invention concerns an oligomer comprising 2 or more than 2 units, wherein each unit comprises a peptidic domain capable of oligomerizing and a domain capable of binding to an acceptor (ligand), wherein the oligomerizing domain is not an antibody or a functional antibody fragment from the constant region.

A functional antibody fragment is, for example, the F_C domain or the constant region of the light or heavy chain.

In a preferred embodiment of the invention the inventive oligomer comprises more than 2 preferably more than 4 units and most preferably the inventive oligomer consist of 5 units.

In another preferred embodiment the individual units oligomerize spontaneously. A peptidic domain capable of oligomerizing is, e.g. a peptide that is known for its tendency to

oligomerize like a pentamerization domain of the Cartilage Oligomeric Matrix Protein (Slavin & Strober (1978) *Nature* **272**, 624-626). Especially preferred units can be separated from the oligomer and re-oligomerized without loss of activity.

In a further preferred embodiment of the invention each of monomeric units has less than 600 amino acids.

The acceptor, to which the inventive oligomer binds, may be of various origin like, for example, any protein that specifically binds to a partner like a substrate, an inhibitor, an activator, an antibody, a receptor and the like. A preferred acceptor is, for example, an antibody or a receptor. Examples for such preferred antibodies or receptors are BCL₁ cells from a B-cell lymphoma surface idiotype or any other receptor expressed on cell surface.

The inventive oligomer may comprise units having the same or different specificities in order to bind to the same or to distinct acceptors; wherein the domain capable of binding to an acceptor can be attached to the C- and/or N-terminus of the oligomerizing domain.

Embraced by the scope of the present invention is also the individual unit capable of oligomerizing as described above, a method for the synthesis of this unit, the expression vector used for this synthesis, as well as the host comprising said expression vector. Preferred hosts are of microbiological origin like *E. coli*.

There are several ways to synthesize the inventive units.

- Via genetic engineering: An expression vector is constructed comprising an expression cassette for the complete inventive unit, that is expressed in a suitable host. Suitable expression cassettes can be prepared by standard techniques in genetic engineering. Beside the information necessary for the synthesis of the desired unit, the expression cassette may additionally contain a signal sequence that forces the secretion of the protein produced. It is also possible to express the inventive unit as a fusion protein with phage coat protein, especially those from filamentous phages and phagemids
- Chemically: Due to the relatively short length of the individual units, they can be synthesized chemically, e.g. via solid phase synthesis.
- Mixed: A part of the inventive unit is expressed by a suitable host and is connected with a chemically synthesized part. For example, the oligomerizing part is synthesized by a

microorganism and is elongated by chemical synthesis to add a domain capable of binding to an acceptor.

The peptidic domain capable of oligomerizing and the domain capable of binding to an acceptor may be connected directly or via a spacer (hinge region) to provide, for example, a greater flexibility of the binding domain. For example, a proline-rich sequence of a spacer is supposed to prevent formation of secondary structure elements and as a consequence a fixed 3-D structure. Moreover, a spacer region like that is generally supposed to be quite rigid due to the conformational constraints of the proline residues, bringing a beneficial effect for the cooperativity of the multivalent binding. Accordingly, in another preferred embodiment of the invention, the peptidic domain capable of oligomerizing and the domain capable of binding to an acceptor are connected via a spacer, that comprises preferably a proline-rich region.

Furthermore, the C-terminus of some or all units of the oligomer may be modified by the addition of a further functional domain, like a marker, an enzymatic or a cytotoxic domain, or a domain that adds additional binding properties, for example to metal atoms or other structural compounds, that can be used, e.g., in affinity chromatography.

The individual units may be connected via disulfide bonds, e.g. spontaneously, or via one or more linker molecules. Such linker molecules are molecules bearing two or more reactive groups like -SH, -N₃, -COOH, -COBr, -COCl, -NH₂, -CHO, -CO-O-CO-, -CO-NH-CO-. Examples are N-5-azido-2-nitrobenzoyloxysuccinimide, p-azidophenazylbromide, p-azidophenyl glyoxal, N-4-(azidophenylthio)phthalimide, bis(sulfosuccinimidyl) suberate, bis-maleimido-hexane, bis[2-(succinimidooxycarbonyloxy)ethyl] sulfone, 1,5-difluoro-2,4-dinitrobenzene, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene, dimethyl adipimidate, dimethyl pimelimidate, dimethyl suberimidate, dithiobis(succinimidylpropionate), disuccinimidyl suberate, disuccinimidyl tatrane, dimethyl 3,3'-dithiobispropionimidate, 4,4'-dithiobisphenylazide, 3,3'-dithiobis (succinimidylpropionate), ethyl-4-azidophenyl-1,4-dithiobutyrimidate, 1-azido-4-fluoro-3-nitrobenzene, N-hydroxysuccinimidyl-4-azidobenzoate, methyl-4-azidobenzoimidate, m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester, N-hydroxysuccinimidyl-4-azidosalicylic acid, p-nitrophenyl-2-diazo-3,3,3-trifluoro propionate, N-succinimidyl(4-azidophenyl)-1,3'-dithiopropionate, sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate, N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate, sulfosuccin-

imidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate, N-succinimidyl(4-iodoacetyl)aminobenzoate, succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, succinimidyl 4-(p-maleimidophenyl)-butyrate, N-succinimidyl 3-(2-pyridyldithio)propionate, bis[2-(sulfosuccinimidooxy-carbonyloxy)ethyl]sulfone, disulfosuccinimidyl tatrane, ethylene glycol-bis(sulfosuccinimidylsuccinate), m-maleimidobenzoyl-N-hydroxysulfosuccinate, sulfosuccinimidyl (4-azidophenyldithio)-propionate, sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate and 2-iminothiolane.

Preferably, oligomerizing units capable of self-assembling are used. The linker molecules mentioned above may be also used additionally to stabilize the oligomer.

As shown in the example, spontaneous pentamerization of COMP assembly domain does not depend on disulfide bond formation, therefore, a preferred embodiment of the inventive oligomer represents a self-assembling molecule able to oligomerize *in vivo* and/or *in vitro*. Thus oligomerization of short peptides bypass folding problems and overcomes expression difficulties previously experienced during oligomerization of relatively complex proteins such as single chain Fv fragments. Moreover, a self-assembling of an oligomer allows it to undergo a reversible denaturation. Thus, heterooligomers can be readily obtained by mixing the inventive units with different specificity (i.e. with different peptide ligands) under denaturing conditions followed, e.g., by dialysis against any physiological buffer. This intrinsic property of the inventive oligomers opens an easy way to produce a chelating oligomer where two or more peptides directed against different epitopes on the same target molecule would be assembled together into a heteropentameric oligomer, allowing to benefit from the power of chelating effect, as it is recently demonstrated for the single chain Fv fragments. The same procedure can be used to produce heterooligomers with affinity to two or more receptors.

Binding studies of ^{125}I labeled Pab-S on BCL1 cells, for example, reveal an avidity of about 1 nM for the surface idiotype which is 2×10^5 fold higher compared to IC_{50} of the peptide S itself (200 μM). Pab-S can be reversibly denatured (4M, urea 95°C, 15 min), regaining full binding activity after renaturation by dialysis in PBS. Data show that Pab-S has remarkable thermostability, retaining the binding activity even after autoclavation (120°C, 20 min).

Furthermore, it is expected that an oligomer harboring a peptide ligand with a nanomolar intrinsic affinity may attain a femtomolar range of avidity, approaching that of streptavidin-biotin interaction

Based on the properties of the inventive compounds, there are several applications for the inventive oligomers, that are all part of the invention.

- Based on the equilibrium binding results of the example Pab-S it is evident that inventive oligomers are excellent molecules for eukaryotic cell, bacteria or viruses targeting; and especially for cell targeting. As it is shown for Pab-S, the avidity for the cell-surface immobilized receptor is much greater than the avidity for the soluble form of the same receptor. This property allows, for example, efficient targeting of the B-cell lymphomas, even in the presence of relatively high level of circulating idiotype antibody.
- Based on the major feature of high avidity binding, especially for the surface immobilized antigens, the inventive oligomers can also be used as an inhibitor of protein-protein interactions, especially those occurring on the cell surface and in solution.
- Heteropentamers can be produced by reassociating two or more different units of the inventive oligomers together. These heterodimers can bind to different epitopes on the same or different molecules and, e.g., can be used as a chelating and/or crosslinking agent.
- The C-terminus of the molecules can be modified easily, e.g. by introducing peptide ligands with second specificity, a cytotoxic tail, a marker to identify acceptors, or a His-tail to chelate different toxic drugs (e.g. heavy metals) in order to deliver them to the target cells where they can be internalized, via an internalized surface receptor (e.g. immunoglobulin receptor) and kill the target cells.
- It is possible, for example, to isolated a panel of C1q specific peptide ligands which can be used to produce a bispecific form of the inventive oligomers capable of C1q fixation and complement activation or complement inhibition.
- Fusion of the peptide ligands to an Fc receptor results in a Nt-Ct bispecific form of the inventive oligomers capable of imitating the function of an Fc region from immunoglobulin.
- Frameworks of the inventive oligomers can also be used to create random peptide libraries displayed on the filamentous bacteriophages. This allows rapid selection of the

phage displayed inventive oligomers which then can be produced in a soluble form, in a suitable host.

- Due to the relatively short length of the monomer polypeptide chain, the inventive oligomers can be synthesized chemically (e.g. standard Fmoc peptide chemistry, synthesis starts from the C-terminus). The N-terminal position of the peptide ligands allows to synthesized first the core molecule (e.g. the pentamerization domain and a linker) and then split the sample and continue synthesis with different peptide sequences on the N-terminus.
- The same principle of chemical synthesis can be used to produce synthetic peptide library displayed on the inventive oligomers. Namely, synthesizing the N-terminal peptides at random (e.g. by Fmoc chemistry as described above), one can obtain a library of the peptides displayed on the inventive oligomer framework. This library can be absorbed on the target molecules or cells, eluted, purified (e.g. using 6xHis tag) and the N-terminal peptide can be sequenced.
- Furthermore, a framework of the inventive oligomers can be used to oligomerize peptides representing known B-cell epitope in order to obtain an efficient vaccines. Addition of different peptides known to enhance the immune response (such as T-helper epitopes or CR binding peptides derived from C3d) is also possible.
- The inventive units can be expressed directly in a genetically engineered multicellular organism, in order to provide an *in vivo* production of the inventive oligomers.
- The inventive units can be expressed *in vitro* using established transcription-translation systems.
- The inventive units may be used *in vitro* as one of the binding reagents in enzyme immunoassays.
- The inventive units can be used to induce apoptosis.
- Due to the solubility and the high specificity of the compounds they can be used in gene therapy, e.g., to target certain receptors for activation or deactivation; or, if connected to toxic compounds, for their destruction.
- Genes encoding the inventive units may be intracellularly expressed in order to inhibit the binding of transcription factors or gene regulating molecules as well as inhibit enzymatic activities.
- The inventive units may be used to inhibit enzymatic activity or adhesion in order to prevent tumor metastization.

- Furthermore, the epitopes of the inventive units can be used for vaccine development.
- Due their high tendency for oligomerization the Cartilage Oligomeric Matrix Protein can be used generally for the pentamerization of low molecular weight compounds or peptides that are not part of the Cartilage Oligomeric Matrix Protein.

Examples

Peptide synthesis. Peptides are synthesized using standard Fmoc solid phase chemistry on an Applied Biosystems 431A peptide synthesizer, lyophilized, re-dissolved in 50% acetic acid, purified by gel filtration through a G-25 sephadex column and analyzed by mass spectroscopy. For competition studies peptides are dissolved in PBS, adjusted to neutral pH, and peptide concentrations are determined by the method of Waddell based on an absorption difference at 215 and 225 nm.

Bacterial strains. *E. coli* TG1 (Wertman *et al.*, (1986) *Gene* 49, 253-262) is used for propagation of plasmids and phages and *E. coli* SG13009 (QIAEX) is used for production of fusion proteins.

Labeling. Typically, Pab-S (17 µg, 0.2 nmol), B1 IgG (75 µg, 0.5 nmol) or B1 Fab' (50 µg, 1 nmol) are labeled in PBS with 100 µCi (1 µCi = 37 MBq) of ¹²⁵I in a in Iodo-Gen (Biorad, 10 µg) coated tubes for 20 min (2 h for Pab-S) at 4°C. Uncoupled iodine is removed by gel filtration on a PD-10 column (Pharmacia). About 40% of the radioactivity for Pab-S and 70% for B1 IgG and B1 Fab' is recovered. Specific activity ranges from 70 to 200 µCi/nmol.

RIA competition on plastic. Polyvinil 96-well plates are coated with BCL1 IgM at 3 µg / ml in PBS for 16 h at 4°C and blocked with 2% MPBS for 1 h at 37°C. Different amounts of peptides are incubated with 10 nCi of ¹²⁵I labeled B1 IgG for 1 h at 37°C. The wells are washed with PBS containing 0.1% of Tween-20 and the amounts of radioactivity retained is measured with an automated multichannel Cobra II γ-counter.

Example 1: Selection of peptides specific for BCL1 surface immunoglobulin receptor.

Cells and antibodies. The BALB/c derived B cell lymphoma BCL₁ (Efimov *et al.*, (1994) *FEBS Letters* 341, 54-58), and the mouse hybridoma B1, secreting an anti-idiotypic monoclonal IgG₁ antibody (B1 IgG), are kindly provided by Dr. Kris Thielemans (Medical

School, VUB, Brussels, Belgium). BCL₁ cells are propagated in BALB/c mice using i.p. injection of 10⁶ cells. The BCL₁ soluble IgM is purified from the serum of a splenomegalic mouse by precipitation with 50% ammonium sulfate, followed by anion exchange and gel filtration chromatography (Mono Q and Superdex 200, Pharmacia). The B1 IgG is purified by protein G-Sepharose (Pharmacia). Fab' fragments are obtained by limited digestion with pepsin, followed by reduction and alkylation, using standard methods.

Peptide selection. Peptide ligands specific for mouse B-cell lymphoma (BCL₁) idiotype, selected from three different peptide libraries displayed on two filamentous bacteriophages libraries of about 10⁷ independent members displaying random hexapeptides called Smith (Scott & Smith (1990) *Science*, **249**, 386-390), and Doorbar (Doorbar & Winter (1994) *J. Mol. Biol.*, **244**, 361-369), are used. In addition a combinatorial library of about 10¹² independent members displaying a tandem of random decapeptides, called Fisch (Fisch *et al.*, *Proc. Natl. Acad. Sci. USA* (1996), **93**, 7761-7766), is used.

Screening of the phage display libraries is performed essentially as described by Fisch *et al.*, (*Proc. Natl. Acad. Sci. USA* (1996), **93**, 7761-7766). Briefly, the purified BCL₁ IgM is coated on immunotubes (Nunc) at 50 µg/ml in PBS (50 mM phosphate, 150m M NaCl, pH 7.4). About 10¹² colony forming units (c.f.u.) of phage from each library are used for each round of selection. After three rounds of panning, individual clones are isolated, and binding of phage to BCL₁ IgM coated on plastic (3µg/ml) is measured by ELISA (Barrett *et al.*, (1992) *Anal. Biochem.* **204**, 357-364) using horseradish peroxidase conjugated anti-M13 antibody (Pharmacia). Plates coated with mouse IgG or with human IgM, are used as negative controls. Specific inhibition of phage binding to BCL₁ IgM, is performed by addition of B1 IgG at 100 µg/ml. The DNA fragments encoding the selected peptides are amplified by PCR and sequenced as described Fisch *et al.*, (*Proc. Natl. Acad. Sci. USA* (1996), **93**, 7761-7766).

Specific phages are selected on the purified BCL₁ IgM, individual phage clones are isolated, and the DNA fragments encoding peptides are amplified by PCR and sequenced (Table 1). Interestingly, two distal Cys residues, potentially capable of forming a disulfide bonded loop, are found in the hexapeptide selected from the Smith library. Idiotype specificity of selected

peptides is demonstrated by inhibition of phage binding with the anti-idiotypic antibody, B1 IgG.

Table 1 Amino acid sequences of selected idiotypic specific peptide ligands for BCL1 immunoglobulin receptor.

Library / sequence *	Selected peptides [†]	Sequ ID	name
Doorbar / X6 - PNDKYE - g3p	SVWRWLPYDKYE [‡]	1	D
Smith / ADGA - X6 - g3p	ADGACRNPWC	2	S
Fisch / X10 - ALLRY - X10 - g3p	TAAGLCEFDQALLRYTCPT [¶]	3	F

* Names and amino acid sequences of the phage displayed peptide libraries; X6 and X10 denotes random hexa- and deca-peptide correspondingly.

[†] Peptides selected from random libraries are in bold type, framework residues are in normal type, corresponding synthetic peptides are in italic type.

[‡] An unusual N to Y substitution in the constant part of the peptide from Doorbar library is observed.

[¶] First ten amino acid of the peptide selected from Fisch library was synthesized.

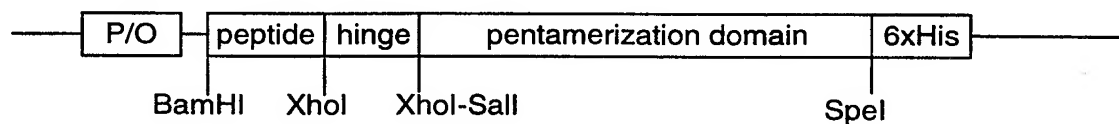
Synthetic peptides corresponding to the selected sequences are prepared as outlined in Table 1. Cysteins containing peptide S is completely oxidized on air losing two hydrogen atoms, as shown by Mass Spectroscopy, which is consistent with the cyclization of the peptide. All synthetic peptides are tested for binding inhibition of ¹²⁵I labeled B1 IgG to BCL₁ IgM coated on plastic. The D and the S peptides inhibit binding with an IC₅₀ of about 60 μ M and 200 μ M, respectively. No inhibition is seen with peptide F up to 2mM, which is used, thereafter, as a negative control. Interestingly, substitution of Cys residues by Ser in peptide S results in a 10 fold loss of affinity, suggesting that disulfide bounded turn conformation of the peptide is favorable for binding to BCL₁ IgM idioype.

Example 2: Pentabody. Molecular design and gene construct

Pentabody fusion protein, is designed consisting of four distinct parts.

- First, a selected peptide specific for BCL₁ IgM idioype represented the N-terminal binding domain.

- Second, a 24 amino acid sequence selected from a long camel Ig hinge region (Hamers-Casterman *et al.*, (1993) *Nature* **363**, 446-448) is placed to provide a space necessary for multivalent binding.
- Third, the hinge region is followed by a 55 amino acid long pentamerization domain, a modification of the described coil-coiled COMP assembly domain (Slavin & Strober (1978) *Nature* **272**, 624-626), known to spontaneously form a five-stranded α -helical bundle. Two cystein residues present at the C-terminal of the wild type sequence allowed the formation of inter-chain disulfide bridges. Based on the recently described model of COMP assembly domain (Kajava (1996) *Proteins* **24**, 218-226) two substitutions Lys29Cys and Ala30Cys are introduced in the modified version, making it possible the formation of additional disulfide bonds near the N-terminal part of the assembly domain in order to further stabilize the α -helical bundle.
- Fourth, a sequence encoding six histidine residues is placed at the C-terminus of the fusion molecule to facilitate the protein purification *via* the metal-chelating affinity chromatography.



The chimeric genes coding for the different Pab are constructed as designed (see below) and named Pab-S, Pab-D and Pab-F, where the last letters stay for the S, D, and F peptide, respectively (see table 1).

Essentially, the DNA sequences encoding the BCL₁ idiotype specific peptides, as well as the hinge region, are assembled from oligonucleotide duplexes. The COMP pentamerization domain is amplified by PCR simultaneously introducing point mutations, and the fusion genes are assembled into a modified pDS-78 expression vector.

Plasmid construction. The construction of plasmid p3bCOMP encoding a 64 amino acid COMP assembly domain is described in (Slavin & Strober (1978) *Nature* **272**, 624-626). All further constructs are made using standard methods of DNA manipulation (Sambrook *et al.*, (1990) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY)). The DNA fragment encoding the COMP domain (residues 26-80, amino acids numbering as in Slavin & Strober (1978) *Nature* **272**, 624-626) is amplified by PCR from

p3bCOMP template using COMP-Xho-BACK (5'-AG ATC CTC GAG GGT GGA GAC TGC TGC CCA CAG ATG CTT AGA; SEQ ID NO 4) and COMP-Spe-FOR (5'GC ACT AGT AGA ACC GCC ACC CGG GGT; SEQ ID NO 5) primers. Thus the resulting product contains two amino acid substitutions Lys29Cys and Ala30Cys as well as XhoI and SpeI sites at the 5' and 3' end, respectively. The DNA duplexes encoding the peptides S, F, and D, are prepared by annealing the oligonucleotides S-up (5'GA TCC GCT GAC GGC GCT TGC CGT ACC CCG TGG TGC GGT C; SEQ ID NO 6) with S-low (5'TC GAG ACC GCA CCA CGG GTT ACG GCA AGC GCC GTC AGC G; SEQ ID NO 7), F-up (5'GA TCC ACT GCT GCA GGT CTG TGC GAA TCC GAC CAG C; SEQ ID NO 8) with F-low (5'TC GAG CTG GTC GAA TTC GCA CAG ACC TGC AGC AGT G; SEQ ID NO 9) and D-up (5'GA TCC TCT GTT TGG CGT TGG CTG CCG TAC GAC AAA TAC GAA C; SEQ ID NO 10) with D-low (5'TC GAG TTC GTA TTT GTC GTA CGG CAG CCA ACG CCA AAC AGA G; SEQ ID NO 11). All three duplexes contain BamHI and XhoI cohesive ends on 5' and 3' end, respectively. By means of three-part ligation, the duplexes encoding peptides and the PCR amplified COMP domain, restricted with XhoI and SpeI enzymes, are joint together in the pDS78 vector, linearized with BamHI and SpeI enzymes, in front of a 6 histidine tail present in the vector (Stuber *et al.*, in *Immunological Methods*, eds. Lefkovits, I. & Perris, B. (Academic Press, New York), pp.121-152). This generated pSC6H pDC6H and pFC6H plasmids which encode S, D and F peptides respectively. A DNA fragment encoding the 24 amino acid hinge region $[(PQ)_2PK(PQ)_4PKPQPK(PE)_2]$, called PX, is prepared by annealing of PX-up (5'TC GAG CGG CAG CCG CAG CCG AAA CCG CAG CCG CAG CCG CAG CCG CAG CCG AAA CCG CAG CCG AAA CCG GAA CCG GAA G; SEQ ID NO 12) and PX-low (5'TC GAC TTC CGG TTC CGG TTT CGG CTG CGG TTT CGG CTG CGG CTG CGG CTG CGG CTG CGG TTT CGG CTG CGG C; SEQ ID NO 13) oligonucleotides encoding the plus and minus strands of the duplex and contain XhoI and Sall cohesive ends on 5' and 3' end, respectively. The PX duplex is ligated into XhoI linearized, dephosphorylated pSC6H pDC6H and pFC6H plasmids, to generate the expression vectors pSPXC6H (DSM 11236), pDPXC6H and pFPXC6H, respectively. The final constructs are verified by dideoxynucleotide sequencing using Sequenase 2.0 (United States Biochemical).

Example 3: Expression and purification of pentabodies.

Pab fusion proteins are expressed in *E. coli* SG13009. The cultures are grown on a shaker at 37°C up to OD₆₀₀ ~0.5, then 1 mM IPTG is added to induce protein synthesis, followed by

further 4 h incubation at 30°C. Bacteria are collected by centrifugation (8000 x g, 15 min, 4°C) and frozen at -70°C. Bacterial pellet is resuspended in PBS pH 7.4, 1mM EDTA, 1mg/ml lysozyme, incubated for 30min at room temperature and subjected to three rounds of freezing/thawing (liquid nitrogen/37°C). The lysate is incubated for 15 min at RT with 0.1mg/ml of DNase I and after centrifugation (23,000 x g, 15 min, 4°C) the supernatant is collected. Imidazol is added to a final concentration of 5 mM and the recombinant protein is absorbed on 2ml of Ni-NTA resin (QIAGEN), equilibrated in 5 mM imidazol, PBS pH 7.4. After extensive wash with PBS containing 5 mM and 20 mM imidazol, retained proteins are eluted with PBS containing 250 mM imidazol. After extensive dialysis against PBS, 1 mM EDTA, proteins are concentrated 5 times with Centriprep 10 concentrator (Amicon), aliquoted and stored at -20°C.

The Pab-S and Pab-F fusion proteins are expressed at high levels (> 30 mg/l), allowing efficient one step metal-chelating affinity chromatography under native conditions. The amount of soluble Pab-D protein is lower than that of Pab-S and Pab-F.

Affinity purified Pab-S and Pab-F molecules are fractionated by FPLC gel filtration. A single elution peak corresponding to a protein of about 85 kDa is observed for Pab-S, whereas two major elution peaks corresponding to the proteins of about 90 and 180 kDa are observed for Pab-F. The high molecular weight peak presumably resulted from dimer formation *via* unsaturated cystein present in peptide F. In both cases, fractions of the elution peak corresponding to a 85-90 kDa protein are collected, pooled and used for binding studies.

Example 4: Equilibrium binding studies.

Cell dilution experiments and homogenous competition curves (i.e. competition for the cell binding between the labeled and the unlabeled forms of the same ligand) are described by a monovalent equilibrium model. The free (F) and bound (B) radioactivity is corrected from the non-immunoreactive fraction (NIF) and non-specific binding (NSB) respectively. Both are expressed in mol/l, taking into account the specific activity of each isotopic dilution (B and F, respectively). The parameters K_d , R, NSB and NIF, where R is the molarity of binding sites and K_d is the dissociation equilibrium constant, are fitted by non-linear regression of the corrected Scatchard equation

$$\frac{B - NSB}{F - NIF} = \frac{R}{Kd} - \frac{(B - NSB)}{Kd}$$

to the experimental data.

To determine maximal immunoreactivity, 125 I-labeled Pab-S, B1 IgG or B1 Fab' (20 nCi) are incubated with serial dilutions of freshly harvested BCL1 cells (0.3 to 100×10^5 cells per $100 \mu\text{l}$). For competition assays, the labeled compounds are incubated with serial dilution of unlabeled competitors and BCL1 cells (25 , 50 or 100×10^5 per $100 \mu\text{l}$). Comparative experiments are performed the same day with the same batch of cells, in 96-wells plates, in triplicates, in a final volume of $150 \mu\text{l}$ of PBS supplemented with 1mg/ml of Bovine Serum Albumin (BSA), at 4°C , under agitation and for 2.5 h. After centrifugation, the amounts of both free and bound 125 I are measured as above from an aliquot of the supernatant and of the pellet washed once with PBS (4°C).

In preliminary solid phase competition assay Pab-S is found to compete the binding of 125 I labeled B1 IgG to BCL₁ IgM idiotype coated on plastic. However, since spatial arrangement of target molecule on the surface may influence multivalent binding, the equilibrium binding parameters of Pab-S are determined directly on live BCL₁ cells, which represent a more relevant biological surface. Purified Pab-S is labeled with 125 I and is shown to bind to BCL1 idiotype on the cell surface. It can be competed by unlabeled Pab-S, B1 IgG, and by a much higher concentration of peptide S, but not by the control Pab-F pentamer. The competitions of 125 I- labeled Pab-S binding by unlabeled Pab-S at 3 different cell concentration and a cell dilution experiment are used together for curve fitting to obtain the equilibrium binding parameters.

The apparent equilibrium binding constant of Pab-S is found to be similar to that of B1 IgG (around 1nM) which represents a 2×10^5 fold increase in avidity compared to the peptide S itself (IC_{50} around $200 \mu\text{M}$). As control, the same binding experiments are performed with 125 I labeled B1 IgG and B1 Fab' fragment. The obtained constants are consistent with already published data. The Scatchard equilibrium binding constants are:

Fragment	Kd [nM]
Pab-S	1.0 ± 0.4
IgG	0.8 ± 0.4

Fab'	3.5 ± 0.1
------	---------------

The lower molarity of binding sites found for Pab-S compared to B1 IgG and Fab provides evidence for a multivalent nature of Pab-S binding.

In another series of experiments we compare the capacity of soluble BCL₁ IgM to compete the binding of either Pab-S or B1 IgG to BCL₁ cells. The results showed that much higher concentration of soluble BCL₁ IgM is needed to compete for Pab-S as compared to B1 IgG (100 vs 4 nM for 80% displacement). These data indicate a higher avidity of Pab-S for the cell surface-immobilized BCL₁ IgM, compared to the soluble BCL₁ IgM. Importantly, no inhibition of Pab-S binding to BCL₁ IgM idiotype is observed in the presence of up to 30 % (v/v) of human or mouse BALB/c serum.

Example 5: Biochemical characterization of Pab-S chimeric protein.

The concentration of purified Pab-S is determined by the method of Waddell, based on an absorption difference at 215 and 225 nm as well as by Bradford protein assay (Bio-Rad). Pab-S molecule is characterized using FPLC gel filtration, SDS-PAGE and CD spectrometry. Oxidized form of Pab-S with inter-chain disulfide bonds is formed by air oxidation during the purification procedure and dialysis against PBS. Completely reduced form is obtained by incubation with 100 mM DTT at 37°C for 30 min, followed by extensive dialysis against PBS, 1mM EDTA, 1mM β-mercaptoethanol.

Under non-denaturing conditions, a single elution peak, corresponding to a protein of about 85 kDa is detected for both oxidized and reduced forms of Pab-S after gel filtration on Superdex G200 column (equilibrated in PBS, 1 mM EDTA, ± 1mM β-mercaptoethanol, elution is monitored at 280 nm with an integrated UV detector (the proteins are analyzed on 10-15% gradient). Under denaturing conditions (Pab-S is denatured by boiling for 20 min in 4 M urea and 100 mM DTT and renatured by extensive dialysis against PBS, 1mM EDTA, 1mM β-mercaptoethanol) in SDS-PAGE, a major protein with apparent molecular weight of about 85 kDa is observed for the oxidized form of Pab-S molecule, whereas a protein with apparent molecular weight of about 17 kDa is observed for the reduced form of Pab-S, in agreement with a covalent pentameric structure. A minor band with apparent mobility of about 68 kDa observed for the oxidized Pab-S corresponds to a partially reduced pentamer,

where only four chain out of five are covalently link by disulfide bridges. From the analysis of the CD spectrum of Pab-S pentamer an α -helical content of about 54 % is determined by fitting the experimental data with a set of reference proteins as described in Vogel (Biochemistry (1987), **26**, 4562-72). This is in a good agreement with the length of the α -helical coiled-coil domain within the entire molecule. Taken together these data indicate that Pab-S molecule is a stable homopentamer of about 85 kDa with a monomer subunits of about 17 kDa held together by in α -helical coiled-coil bundle, where the five chains are covalently crosslinked by disulfide bonds.

It is known that coiled coil structures can undergo reversible denaturation. Indeed, the Pab-S molecule is denatured in urea at 95°C under reducing conditions and refolded into the pentamer by simple dialysis against PBS, as demonstrated by FPLC gel filtration of renatured Pab-S labeled with ^{125}I . Noteworthy, renaturation of Pab-S restores full binding activity as shown by binding competition on BCL1 cells.

To visualize the spatial arrangement of Pab-S molecular modeling of its three-dimensional structure is undertaken. Pab-S molecule is modeled using the computer graphics program TOURBO-FRODO (Roussel & Cambillan (1989) in *Silicon Graphics Geometry Partner Directory (Fall 1989)*, eds. Silicon Graphics (Silicon Graphics, Mountain View, CA), pp.77-78). The structure is further refined by the X-PLOR program version 3.1 (Brunger. (1992) in X-PLOR version 3.1 A system for X-ray Crystallography and NMR, (New Haven, Yale University Press)) using the following procedure: a 5 ps molecular dynamics simulation at 300 K and then 1,000 steps of conjugate gradient minimization, carried out with $1/r$ dependent dielectric constant.

The stereochemical analysis shows that frequently occurring type I conformation of β -turn allows formation of disulfide bond between the two cysteins of peptide S. This conformation is chosen, as the most likely, for the peptide ligand. For the hinge region a molecular dynamic simulation with subsequent energy minimization resulted in a set of possible conformations. Out of it, a "good-looking" conformation is arbitrary chosen. Finally, previously modeled five-stranded α -helical coiled-coil structure is taken for the pentamerization domain. The analysis suggests a fivefold symmetrical structure of Pab-S protein. The analysis of the modeled Pentabody structure shows that such a molecule

should be capable of simultaneous binding to five surface receptor, provided they are located up to 80 Å apart from each other. This criterion is largely satisfied in the case of the surface immunoglobulin receptor, whose variable domains can be spaced at about 50 Å, judging by their van der Waals contact radii.

Example 6: Use of pentabody in *in vitro* for assays

To measure the level of an antigen of interest such as prostate specific antigen (PSA) which is an enzyme of the kallikrein family, peptides displayed on a phage display library may be screened for those binding specifically the antigen of interest, such as PSA, with a great specificity and avidity. Screening may be as described in the examples above, particularly example 1. Pentabody expressing the selected peptide(s) in pentameric form may be constructed, expressed and purified as described in the examples above, particularly examples 2 and 3. The selected pentabodies may be labeled with a marker enzyme such as peroxidase.

Antibodies specific for the antigen of interest, such as PSA, may be immobilized on a solid phase such as plates or polystyrene balls. The immobilized, unsolubilized antibodies may be incubated with the test fluid, such as serum or other biological fluid for the first step of immunoabsorption. After incubation, the solid phase may be washed.

The selected pentabody labeled with a marker enzyme may be incubated with the immunoabsorbed antigen, such as PSA. Then the substrate for the marker enzyme conjugated to the pentabody and a chromogen may be added. The optical density may then be measured. The optical density of the chromogen will be proportional to the amount of antigen, such as PSA, present. The assays of the invention may also comprise radioimmunoassays using the pentabodies of the invention, the above approach and methods known in the art.

Deposition of Microorganisms

The following microorganisms are deposited according to the Budapest Treaty with the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig:

E. coli SG13009 pSXC6H

21.10.1996

DSM11236

Sequenzlisting

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: CIBA-GEIGY AG
- (B) STREET: Klybeckstr. 141
- (C) CITY: Basel
- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 4002
- (G) TELEPHONE: +41 61 696 11 11
- (H) TELEFAX: + 41 61 696 79 76
- (I) TELEX: 962 991

(i) APPLICANT:

- (A) NAME: University of Lausanne
- (B) STREET: Chemin des Boveresses 155
- (C) CITY: Epalinges
- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 1066
- (G) TELEPHONE:
- (H) TELEFAX:
- (I) TELEX:

(i) APPLICANT:

- (A) NAME: Swiss Institute for Allergy and Astma Research
- (B) STREET: Obere Stasse 22
- (C) CITY: Davos
- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 7270
- (G) TELEPHONE:
- (H) TELEFAX:
- (I) TELEX:

(i) APPLICANT:

- (A) NAME: ISREC
- (B) STREET: Chemin des Boveresses 155
- (C) CITY: Epalinges

- 20 -

- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 1066
- (G) TELEPHONE:
- (H) TELEFAX:
- (I) TELEX:

(ii) TITLE OF INVENTION: Oligomers

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser	Val	Trp	Arg	Trp	Leu	Pro	Tyr	Asp	Lys	Tyr	Glu
1				5						10	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- 21 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala	Asp	Gly	Ala	Cys	Arg	Asn	Pro	Trp	Cys
1				5					10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Thr	Ala	Ala	Gly	Leu	Cys	Glu	Phe	Asp	Gln	Ala	Leu	Leu	Arg	Tyr	Thr
1				5					10					15	

Cys Pro Thr

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGATCCTCGA GGGTGGAGAC TGCTGCCCAC AGATGCTTAG A

41

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- 22 -

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCAC TAGTAG AACCGCCACC CGGGGT

26

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GATCCGCTGA CGGCGCTTGC CGTACCCCGT GGTGCGGTC

39

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCGAGACCGC ACCACGGGTT ACGGCAAGCG CCGTCAGCG

39

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GATCCACTGC TGCAGGTCTG TGCGAATCCG ACCAGC

36

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCGAGCTGGT CGAATTCGCA CAGACCTGCA GCAGTG

36

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATCCTCTGT TTGGCGTTGG CTGCCGTACG ACAAATACGA AC

42

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCGAGTTCGT ATTTGTCGTA CGGCAGCCAA CGCCAAACAG AG

42

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TCGAGCGGCA GCCGCAGCCG AAACCGCAGC CGCAGCCGCA GCCGCAGCCG AAACCGCAGC

60

CGAAACCGGA ACCGGAAG

78

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
TCGACTTCCG GTTCCGGTTT CGGCTGCGGT TTCGGCTGCG GCTGCGGCTG CGGCTGCGGT    60
TTCGGCTGCG GCTGCGGC                                                    78
```

Claims

1. An oligomer comprising 2 or more than 2 units, wherein each unit comprises a peptidic domain capable of oligomerizing and a domain capable of binding to an acceptor, wherein the oligomerizing domain is not an antibody or a functional antibody fragment from the constant region.
2. An oligomer according to claim 1 comprising more than 4 units.
3. An oligomer according to claim 1 consisting of 5 units.
4. An oligomer according to claim 1, wherein the acceptor is a antibody or a receptor.
5. An oligomer according to claim 1, wherein each of said units has less than 600 amino acids.
6. An oligomer according to claim 1, wherein the peptidic domain capable of oligomerizing and the domain capable of binding to an acceptor are connected via a spacer (hinge region).
7. An oligomer according to claim 6, wherein the spacer comprises a proline-rich region.
8. An oligomer according to claim 1, wherein at the C-terminus of some or all units a further functional domain is attached.
9. An oligomer according to claim 1, wherein the individual units oligomerize spontaneously.
10. Use of the pentamerization domain of the Cartilage Oligomeric Matrix Protein for the pentamerization of low molecular weight compounds or peptides that are not part of the Cartilage Oligomeric Matrix Protein.
11. An oligomer according to claim 1, wherein the oligomerizing domain is the pentamerization domain of the Cartilage Oligomeric Matrix Protein.
12. An oligomer according to claim 1 comprising units that bind to distinct acceptors.
13. A unit capable of oligomerizing according to claim 1.

14. Use of an oligomer according to claim 1 for identification and/or marking of acceptors.
15. Use of an oligomer according to claim 1 for eukaryotic cell, bacteria or viruses targeting.
16. Use of an oligomer according to claim 1 for cell targeting.
17. Use according to claim 16 for targeting of B-cell lymphomas.
18. Use of an oligomer according to claim 1 for the inhibition of protein-protein interactions.
19. Use of an oligomer according to claim 12 as a chelating agent.
20. Use of an oligomer according to claim 12 as a crosslinking agent.
21. Use of oligomers according to claim 1 for the construction of libraries.
22. Use of oligomers according to claim 1 for the induction of apoptosis.
23. Use of oligomers according to claim 1 for the intracellular inhibition of transcription factor binding, gene regulating molecules and/or enzymatic activities.
24. Use of oligomers according to claim 1 for prevention of tumor metastatization.
25. Use of oligomers according to claim 1 *in vitro* as one of the binding reagents in an enzyme immunoassay.
26. Use of oligomers according to claim 1 *in vitro* as one of the binding reagents in radioimmunoassays.
27. Method for the synthesis of a unit according to claim 13.
28. An expression vector for the synthesis of a unit according to claim 13.
29. A host, comprising an expression vector for the synthesis of a unit according to claim 13.
30. A microbiological host, comprising an expression vector for the synthesis of a unit according to claim 13.
31. A method for the production of an oligomer according to claim 1.

32. An oligomer according to claim 1, wherein the epitopes are used for vaccine development.

EP 97/05897


BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Ciba-Geigy AG
CH-4002 Basel

Institute of Biochemistry
University of Lausanne
155, Chemin de Boveresses
CH-1066 Epalinges

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: SG13009/pSPXC6H	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 11236
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>(X) a scientific description (X) a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1996-10-21 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1996-10-22

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

EP 97/05897


INTERNATIONAL FORM

Ciba-Geigy AG
CH-4002 Basel

Institute of Biochemistry
University of Lausanne
155, Chemin de Boveresses
CH-1066 Epalinges

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
<p>Name: Ciba-Geigy AG CH-4002 Basel</p> <p>Address: Institute of Biochemistry University of Lausanne 155, Chemin de Boveresses CH-1066 Epalinges</p>	<p>Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 11236</p> <p>Date of the deposit or the transfer¹: 1996-10-21</p>
III. VIABILITY STATEMENT	
<p>The viability of the microorganism identified under II above was tested on 1996-10-21². On that date, the said microorganism was</p> <p>(X)³ viable</p> <p>()³ no longer viable</p>	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
<p>Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH</p> <p>Address: Mascheroder Weg 1b D-38124 Braunschweig</p>	<p>Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):</p> <p></p> <p>Date: 1996-10-22</p>

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/EP 97/05897

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 26985 A (UNIV TEXAS) 12 October 1995 see page 9, line 5 - page 24 ---	1,4,5,9, 12-16, 18,23-31
X	WO 95 31540 A (MEDICAL RES COUNCIL ;H-J. HOPPE; K.B.M. REID) 23 November 1995 see page 19, line 9 - page 20, line 13 ---	1,4,5,9, 12-16, 23-31
A	A.V. KAJAVA: "Modeling of a five-stranded coiled coil structure for the assembly domain of the cartilage oligomeric matrix protein" PROTEINS, vol. 24, no. 2, February 1996, pages 218-226, XP000647810 see the whole document --- -/--	1-16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

27 March 1998

Date of mailing of the international search report

07.04.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/EP 97/05897

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	V.P. EFIMOV ET AL.: "The thrombospondin-like chains of cartilage oligomeric matrix protein are assembled by a five stranded alpha-helical bundle between residues 20 and 83" FEBS LETTERS, vol. 341, 1994, pages 54-58, XP002017642 see the whole document ---	1-16
P,X	WO 96 37621 A (MORPHOSYS GESELLSCHAFT FÜR PROTEINOPTIMIERUNG ; P. PACK ; A. HOESS) 28 November 1996 * paragraph bridging pages 18 and 19 * -----	1-18, 23-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/05897

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 24 and claims 15-20, 22 and 23 insofar they concern an in vivo use, are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the oligomer.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/05897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9526985 A	12-10-95	AU 2207195 A	23-10-95
WO 9531540 A	23-11-95	AU 2451995 A	05-12-95
		CA 2190264 A	23-11-95
		EP 0757720 A	12-02-97
		JP 10500298 T	13-01-98
WO 9637621 A	28-11-96	EP 0827544 A	11-03-98